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***N*-Acylethanolamines as Novel Alcohol Dehydrogenase 3 Substrates**

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N-Acylethanolamines as Novel Alcohol Dehydrogenase 3 Substrates

by

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A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science
Department of Chemistry
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I would like to dedicate this work to my parents, Ljubica and Milorad, and my brother, Goran. Without their love and support I would not be where I am today.

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Milena Ivkovic

ABSTRACT

N-Acylethanolamines (NAEs) are a class of fatty acid amides that act as important mammalian signaling molecules. *N*-Arachidonoyethanolamine is the best-studied representative and is one of the endogenous ligands for endocannabinoid receptors. NAEs play a role in the regulation of appetite, act as anti-inflammatory and analgesic agents, and are thought to have a neuroprotective function as well. They have been proposed to also serve as precursors to *N*-acylglycines (NAGs). *N*-Acylglycinals are likely to be intermediates between the NAEs and the NAGs. The sequential actions of a putative fatty alcohol dehydrogenase and a putative fatty aldehyde dehydrogenase are thought to affect the NAD^+ -dependent oxidation of the NAEs to the NAGs. NAGs, in turn, serve as precursor in the biosynthesis of primary fatty acid amides (PFAMs), another class of mammalian regulatory molecules. Alcohol dehydrogenase 3 (ADH3), an enzyme known to oxidize mid- and long-chain alcohols to aldehydes, was evaluated for its potential in oxidation of NAEs to *N*-acylglycinals.

In order to evaluate the possibility of ADH3 involvement in NAE metabolism, variable chain length NAEs were synthesized and evaluated as substrates for bovine liver ADH3. NAEs were oxidized by ADH3 in the presence of NAD^+ , yielding the corresponding *N*-acylglycinals. V_{Max}/K_M values for assayed NAEs were low relative to cinnamyl alcohol, one of the preferred substrates for ADH3. Our data suggest that the ADH-mediated oxidation of NAEs could occur *in vivo*, but that ADH3 is unlikely to be the *in vivo* catalyst.

Chapter I – Introduction

N-Acylethanolamines (NAEs)

N-Acylethanolamines (NAEs) belong to the class of fatty acid amides, which also includes primary fatty acid amides (PFAMs), *N*-acyldopamines (NADAs), *N*-acylamides (NAMs) and *N*-acylamino acids (NAAs) (1). They are the most widely studied group of fatty acid amides and represent an important class of mammalian signaling molecules. NAEs have been isolated from the brain as well as a variety of other tissues (1), and have been associated with a number of important processes, such as appetite control, regulation of energy metabolism, and anti-inflammatory response (1-3). *N*-Arachidonylethanolamine (anandamide) is an endogenous ligand for cannabinoid receptors (CB1 and CB2), and as a result, it is the most extensively studied of all NAEs. Anandamide plays an important role in the stimulation of appetite, reduction of body temperature and movement, and also has analgesic properties (1-4). It is thought that most of these effects of anandamide are mediated by its binding to CB1 receptor.

Other long-chain NAEs whose functions have been established also play important roles in the body. *N*-Palmitoylethanolamine is a well-known anti-inflammatory agent, and also has analgesic properties (1, 2, 5). *N*-Oleoylethanolamine inhibits food intake through binding to PPAR α (peroxisome proliferator-activated receptor α) and GPR119 (G-protein coupled receptor), thereby acting in opposition to anandamide (1, 3). *N*-Stearoylethanolamine exhibits similar effects as anandamide with regard to analgesia, body temperature and motility, although it is unable to bind to cannabinoid receptors (1). It has also recently been found to possess anti-inflammatory properties (6). Long-chain NAEs, including *N*-stearoylethanolamine and *N*-palmitoylethanolamine, have been thought to simulate the effects of anandamide by acting as “entourage” compounds (7-8). All long chain NAEs are degraded by fatty acid amide hydrolase (FAAH), and “entourage” NAEs compete with anandamide for binding to FAAH, thereby effectively

reducing its rate of degradation and prolonging its effects in the body. NAEs have also been postulated to have a neuroprotective action, as their levels are increased in injured neurons (4). Increase in NAE levels following cell stress and injury has also been found in other organs, such as heart and testes, suggesting they may play a similar role in these tissues as well (2).

Primary route for NAE synthesis is by the action of *N*-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD), which catalyzes the cleavage of *N*-acylphosphatidylethanolamine (NAPE) into NAE and phosphatidic acid (PA) (Fig. 1). NAE degradation to fatty acid and ethanolamine is accomplished primarily through the action of fatty acid amide hydrolase (FAAH), although another enzyme capable of doing the same chemistry, *N*-acylethanolamine-hydrolyzing acid amidase (NAAA), has been discovered recently (9-10) (Fig. 2). Alternatively, it has been proposed that NAEs could act as precursors in the biosynthetic pathway of PFAMs (1, 11), a related class of fatty acid amides (Fig 3).

Figure 1. NAE biosynthesis reaction.

Figure 2. NAE degradation pathway.

Figure 3. Proposed pathway for biosynthesis of PFAMs from NAEs.

PFAMs were first identified in human plasma (12), but have since been isolated from brain and cerebrospinal fluid as well. Like NAEs, they function as signaling and regulatory molecules in the body. The best-studied PFAM is oleamide, which is important in regulation of sleep, memory, body temperature and movement (1). Other

naturally occurring PFAMs include erucamide, linoleamide and elaidamide (1). Initially proposed biosynthetic route to PFAMs was through reaction between fatty acid and ammonia, catalyzed by FAAH (13). However, although this chemistry occurs *in vitro*, it requires relatively high pH and high ammonia concentration, making it unlikely that it is the primary synthetic pathway *in vivo*. There are two other possible routes to PFAM biosynthesis. First route involves cytochrome c-catalyzed amidation of fatty acyl-CoA thioester by ammonia (1, 14-15). The other route is by the action of peptidylglycine- α -amidating monooxygenase (PAM) on *N*-acylglycines (NAG), resulting in production of PFAMs and glyoxylate (Fig. 3). NAGs are well established as good substrates for PAM, but their biosynthetic pathway is unclear. There is evidence that NAEs could serve as precursors to NAGs through the sequential actions of a putative fatty alcohol dehydrogenase (FADH) and a putative fatty aldehyde dehydrogenase (FALDH) (Fig. 3). Support for this pathway comes from experiments in which ^{14}C -labeled *N*-arachidonoylglycine was detected in liver cells that were treated with ^{14}C -labeled anandamide (11). These experiments strongly suggest that there is a set of liver ADH and ALDH enzymes capable of catalyzing the conversion of NAEs to NAGs.

Alcohol Dehydrogenases (ADHs)

Alcohol dehydrogenases (ADHs) comprise a family of NAD^+ -dependent, Zn-containing, dimeric enzymes responsible for oxidation of alcohols to aldehydes. ADH dimers are composed of identical subunits, each containing two Zn ions, one of which plays a structural role, and one which is involved in catalysis. Each subunit is composed of two domains: a coenzyme binding domain, located close to the interface with other subunit and a catalytic domain, located toward other ends of the dimer (Fig. 6). ADH enzymes are divided into six classes based on their structure, substrate preference, sensitivity to inhibitors, and electrophoretic mobility.

Class I ADH (ADH1) enzymes are expressed in all vertebrates, (16) and in humans they are present in the liver, kidney, lung, mucosa of the stomach and lower digestive tract (17). This class contains a number of isozymes, created through the different combinations of three subunits designated as α , β , and γ . Among ADHs, ADH1

Alcohol Dehydrogenase 3 (ADH3)

Class III alcohol dehydrogenase (ADH3) is found in virtually all eukaryotes and prokaryotes and represents the ancestral enzyme from which all the other ADHs originated. It was first isolated from human liver in 1981 (25), and it was fully characterized in 1984 (26). Shortly thereafter, it was recognized that ADH3 is identical to the previously characterized glutathione-dependent formaldehyde dehydrogenase (27-28), which catalyzes the oxidation of *S*-(hydroxymethyl)-glutathione (HMGS) (formed through non-enzymatic conjugation of formaldehyde to glutathione (GSH)) (Fig. 5). Together with *S*-formylglutathione hydrolase, ADH3 forms a major formaldehyde detoxification pathway in the body (Fig 5).

Figure 5. Formaldehyde detoxification pathway.

Ethanol is a very poor substrate for ADH3, and saturation is never observed, precluding the determination of K_M and V_{Max} values. Unlike all the other characterized ADHs, ADH3 is not inhibited by 4-methylpyrazole, and cannot oxidize retinol. It also lacks the aldehyde dismutase activity of ADH1 and ADH2 (18). Besides HMGS, the best substrates for ADH3 are medium- and long-chain alcohols (5 carbons and up), and ω -hydroxy fatty acids, although it also able to oxidize 20-hydroxyleukotriene B4 (29).

This preference for larger substrates can be explained by the differences in the active site size and structure between ADH3 and other ADH enzymes characterized to date. In all ADH enzymes, active site is positioned in the cleft between the coenzyme binding domain and the catalytic domain of the subunit (Fig. 6). In ADH1 and ADH4,

binding of the coenzyme induces a conformational change whereby catalytic unit rotates by $\sim 10^\circ$ toward the coenzyme-binding domain, thereby converting the enzyme from the “open” to the “closed” conformation (30-35). Closure of the active site enables tighter binding for the smaller substrates, accounting for the preference these enzymes have for shorter alcohols. Because NAD^+ binding is required in order to achieve effective substrate binding, these enzymes display an ordered bi-bi mechanism (31-32, 36).

Figure 6. ADH3 dimer (adapted from ref. 30). Subunits are colored blue and green. Coenzyme binding domains are colored light blue and light green, and catalytic domains are dark blue and dark green. Catalytic Zn ions are colored red, and structural Zn ions are shown in grey. Bound NAD^+ molecules are shown in purple. Yellow areas represent important secondary structures in the proximity of the active sites.

In contrast, the ADH3 active site displays a “semi-open” configuration that is in-between the fully “open” and fully “closed” conformations exhibited by ADH1 (30-32). In addition to this, coenzyme binding results in only small conformational changes between the two domains, leaving a large, solvent-exposed active site approximately twice the size of the ADH1 active site (30). Substrate binding is therefore not dependent on prior NAD^+ binding, and, as a result, ADH3 has a random bi-bi mechanism (36). Having a large active site means that ADH3 is capable of accommodating bulky substrates, like long chain alcohols, ω -hydroxy fatty acids and HMGS. It also accounts for its poor activity toward the smaller substrates, such as ethanol, which are unable to form the bonding interactions they make in other ADHs. In addition to the open active

site, smaller substrates lack the stabilization provided by the loop formed by residues 112 to 120 in ADH1, which is replaced by an α -helix in ADH3 which fails to provide any binding support (30).

The well-established preference of ADH3 for large, hydrophobic alcohol substrates suggests that it could act as the putative FADH, catalyzing the oxidation of NAEs to *N*-acylglycinals. It is also the most ubiquitous of all the ADH enzymes, and it is the sole ADH representative so far characterized in the brain (37), where abundance of NAEs is found. Its substrate preference combined with its presence in the tissues where NAEs have been found, led us to investigate ADH3 as a potential FADH, acting in the first step of the proposed PFAM biosynthetic pathway to catalyze the oxidation of NAEs to *N*-acylglycinals (Fig. 3).

Chapter 2 – Materials and Methods

Enzyme Purification

Bovine liver ADH3 was purified following the modified procedure of Pourmotabbed et al. (38). Purification was accomplished by performing ammonium sulfate precipitation, followed by DEAE-cellulose, mimetic blue II, and blue dextran agarose chromatography.

A. Ammonium Sulfate Precipitation

Fresh bovine liver was obtained from Central Packing Co Inc, (Center Hill, FL), cut into parts, and frozen at -80°C . Bovine liver portion weighing 265 g was thawed out, chopped into small pieces, combined with 795 ml of 50 mM potassium phosphate pH 7.5, 5 mM β -mercaptoethanol (BME), and a cocktail of protease inhibitors (1 $\mu\text{g}/\text{ml}$ leupeptin, 1 $\mu\text{g}/\text{ml}$ pepstatin A, and 1 $\mu\text{g}/\text{ml}$ aprotinin), and blended for 3 x 30 seconds in a Waring blender at high setting. Blended mixture was centrifuged in a Sorvall RC 6 centrifuge at $20,000 \times g$ and 4°C for 40 minutes. Pellet was then discarded and supernatant centrifuged at $32,000 \times g$ and 4°C for 30 minutes. Reddish-brown supernatant (670 ml) was obtained following the second centrifugation, and taken to 45% saturation by addition of 173 g of ammonium sulfate. Finely ground ammonium sulfate was added slowly over a 1-hour period with continuous stirring, and pH of the solution was maintained at 7.4 by addition of concentrated NH_4OH . Following salt addition, solution was left to equilibrate for 2 hours without stirring. Equilibrated solution was centrifuged at $47,800 \times g$ for 30 minutes. Recovered supernatant (600 ml) was then taken to 67% saturation by addition of 84 g of ammonium sulfate. As previously, ammonium sulfate was added slowly over 1 hour and pH was maintained by addition of concentrated NH_4OH . Following equilibration, solution was centrifuged at $47,800 \times g$ for 30 minutes. Supernatant was discarded and pellet dissolved in minimum amount of 10 mM Tris pH

7.6 and 5 mM BME solution. Sample was then dialyzed in the same buffer in order to remove the salt.

B. DEAE-Cellulose Chromatography

Following dialysis, sample was loaded onto a DEAE cellulose column (2 x 40 cm) previously equilibrated with 10 mM Tris pH 7.6 and 5 mM BME. Column was washed with running buffer until A_{280} was reduced to 0.2, and eluent was collected in 15 ml fractions. ADH3 was eluted off the column by running a linear pH gradient with 500 ml of running buffer (10 mM Tris pH 7.6) and 500 ml of 10 mM Tris pH 7.0. Fractions were assayed for protein content by measuring absorbance at 280 nm, while enzyme activity was assayed by following NADH production using cinnamyl alcohol as substrate. Fractions with highest specific activity were pooled together and dialyzed into solution of 10 mM Tris pH 8.0 and 2 mM dithiothreitol (DTT).

C. Mimetic Blue II Chromatography

Dialyzed sample was loaded onto a Mimetic Blue II affinity column (1.5 x 20 cm) that was previously equilibrated with 10 mM Tris pH 8.0 and 2 mM DTT. Column was washed with the same buffer and flow rate was adjusted to 1 ml/min. Eluent was collected in 4 ml fractions. Protein content was monitored by measuring absorbance of fractions at 280 nm. Once A_{280} was below 0.2, a linear gradient of 0-20 mM NAD^+ (total volume 200 ml) was applied to the column in order to elute ADH3. Fractions were assayed for enzyme activity by following NADH production at 340 nm, with cinnamyl alcohol as substrate. Fractions with highest specific activity were pooled together and dialyzed into 0.5 mM Tris pH 7.0 and 5 mM BME.

D. Blue Dextran Agarose Chromatography

Following dialysis to remove NAD^+ , sample was loaded onto a Blue Dextran agarose column (5 ml), that had been equilibrated with 0.5 mM Tris pH 7.0 and 5 mM BME. Column was washed with the same buffer and the eluent was collected in 3 ml fractions, which were assayed for protein content and enzymatic activity as described before. Fractions with the highest specific activity were analyzed for purity using SDS-

PAGE, and those containing only a single protein band corresponding to the weight of an ADH3 subunit (~40 kDa) were pooled together. Protein concentration of purified ADH3 was determined using the Bradford assay, and concentrated enzyme was stored at -80 °C.

E. Enzyme Activity Assay

Enzyme activity was evaluated by following NADH production at 340 nm, with cinnamyl alcohol as the substrate. Assays were done in 100 mM glycine pH 10, 5 mM cinnamyl alcohol, and 2.5 mM NAD⁺ at 37 °C. NADH production was followed at 340 nm using JASCO V-530 UV-Vis spectrophotometer. When scanning crude enzyme fractions, which are known to include ADH1 and ADH2, assay also included 3 mM pyrazole, a known inhibitor of these two enzymes. In addition to this, rates for controls without the substrate were obtained and subtracted from the reaction rates in order to eliminate false positives.

Syntheses

A. Synthesis of NAEs

A number of *N*-acylethanolamines were available commercially in high purity and these were obtained and used without further purification. *N*-Arachidonoylethanolamine and *N*-linoleoylethanolamine were purchased from Cayman Chemical Company, *N*-acetylethanolamine was obtained from Sigma-Aldrich, and *N*-propionylethanolamine was purchased from TCI.

The remaining *N*-acylethanolamines were synthesized as described in Jonsson et al. (7-8) (Scheme 1). Briefly, an excess of ethanolamine (~ tenfold) was added to 15-20 ml of anhydrous CH₂Cl₂ and placed in an ice-bath. Acyl chloride (0.012 moles) was added drop-wise to the solution while stirring and under N₂. Upon complete addition of the chloride, reaction mixture was removed from ice and left stirring at room temperature for another 12 hours. Mid- and long-chain NAEs (octanoyl and longer) were purified by recrystallization in ethanol/water. Short-chain NAEs (butyryl, hexanoyl and benzoyl) were purified using a silica column run in ethyl acetate: methanol (80:20). The yields for the synthesized NAEs were as follows: *N*-butyrylethanolamine – 48.3 %, *N*-

hexanoylethanolamine – 20.6 %, *N*-benzoylethanolamine – 28.2 %, *N*-octanoylethanolamine – 44.5 %, *N*-decanoylethanolamine – 77.4 %, *N*-lauroylethanolamine – 77.6 %, *N*-myristoylethanolamine – 73.4 %, *N*-oleoylethanolamine – 84.5 %. Purity and identity of synthesized NAEs were confirmed by ^1H and ^{13}C NMR on iNOVA 400 MHz (see Figures A1-A8). All acyl chlorides and ethanolamine were obtained from Sigma-Aldrich, and used without further purification. Anhydrous CH_2Cl_2 was purchased from EMD.

Scheme 1. Synthesis of NAEs.

B. Synthesis of $[1',2'\text{-}^{13}\text{C}]$ -*N*-Octanoylethanolamine

Uniformly ^{13}C -labeled ethanolamine HCl was dissolved in CH_3CN and a 10-fold molar excess of triethylamine (TEA) under N_2 . One equivalent of octanoyl chloride was added drop-wise, and the reaction mixture was left to stir at room temperature for 12 hours (Scheme 2). Reaction progress was monitored by TLC (ethyl acetate: methanol (80:20 (v/v))). Once reaction was > 90% complete, solvent was removed, and residue was redissolved in the same mobile phase used for TLC. $[1',2'\text{-}^{13}\text{C}]$ -*N*-Octanoylethanolamine was purified by silica column chromatography in 53 % yield, and characterized by ^1H and ^{13}C NMR spectra obtained on the Varian iNOVA 400 (Fig. A-9).

Scheme 2. Synthesis of $[1',2'\text{-}^{13}\text{C}]$ -*N*-Octanoylethanolamine. ^{13}C labeled atoms are marked by *.

C. Synthesis of *N*-Acylglycinals

N-Benzoylglycinal and *N*-octanoylglycinal were prepared using the modified procedure of Brown (39) (Scheme 3). Aminoacetaldehyde diethyl acetal (50 mmoles) was dissolved in 75 ml of diethyl ether and placed in an ice-bath. Next, one equivalent (5 ml of 15 M) KOH was added to the solution. One equivalent of acyl chloride and another two equivalents of KOH were simultaneously added drop-wise to the reaction mixture. Once addition was complete, reaction was left stirring on ice for another 3 hours. Reaction progress was monitored by TLC (hexanes: ethyl acetate (1:1)). Ether and aqueous layers were separated, and ether layer was washed with 5 x 30 ml of saturated NaCl, and dried with MgSO₄. Solvent was removed under vacuum to obtain *N*-acylglycinal diethyl acetal. Product purity and identity were confirmed by ¹H and ¹³C NMR on Burker DPX 250.

N-Benzoylglycinal and *N*-octanoylglycinal were obtained by acid-catalyzed deprotection of the corresponding *N*-acylglycinal diethyl acetals. *N*-acylglycinal diethyl acetal was dissolved in 25 ml of diethyl ether, 10 ml of saturated NaCl, 7 ml of deionized H₂O, and 3 ml of concentrated HCl. Reaction mixture was left stirring for 12 hours, and reaction progress was monitored by TLC (CH₂Cl₂: EtOAc (80:20)). Organic layer was separated and dried with MgSO₄. Solvent was removed under vacuum and the residue was dissolved in CH₂Cl₂: EtOAc (80:20), and applied to a silica column (5 x 20 cm). *N*-Benzoylglycinal and *N*-octanoylglycinal were obtained in 9 %, and 11 % yields, respectively. Purity and identity of both compounds were confirmed by ¹H and ¹³C NMR spectra obtained on Varian iNova 400 MHz (Figs A-10-11).

Scheme 3. Synthesis of *N*-Acylglycinals.

D. Synthesis of *N*-Octanoylglycine

N-Octanoylglycine was synthesized following the modified procedure of Iyer et al. (40). Glycine (0.02 mol) was dissolved in 100 ml of deionized water, and pH was adjusted to 10 by addition of 2 M NaOH. One equivalent of octanoyl chloride was added drop-wise to the solution, while maintaining pH 10 with addition of 2 M NaOH. Following 1.5 hours of reaction time, solution was acidified to pH 1 by addition of 30% H₂SO₄. White precipitate formed following acidification, and was filtered off and dried. Precipitate was then recrystallized with EtOAc/petroleum ether to obtain the pure product

in 49% yield. *N*-Octanoylglycine identity and purity was confirmed by ^1H and ^{13}C NMR on Varian iNOVA 400 (Fig A-13).

E. Preparation of *N*-Octanoylglycinal semicarbazone

N-Octanoylglycinal semicarbazone standard was prepared by reacting *N*-octanoylglycinal with a large excess (~10 fold) of semicarbazide in aqueous solution. *N*-Octanoylglycinal semicarbazone was purified using HPLC and characterized by ^1H and ^{13}C NMR on Varian iNova 400 (Fig A-12).

Kinetic Assays of NAE Substrate Activity with ADH3

A. Spectrophotometric NADH Assay

Kinetic constants for short and mid-length NAEs were determined from initial reaction rates obtained by following NADH production at 340 nm. Reaction conditions were as follows: 100 mM glycine pH 10, 2.5 mM NAD^+ , and 15-30 $\mu\text{g/ml}$ ADH3 in 1 ml total reaction volume. NAE concentrations were between $0.1K_M$ and $10K_M$. Enzyme assays were conducted at 37 °C, using JASCO V-530 UV-Vis spectrophotometer. Glycine was purchased from J.T. Baker, sodium pyrophosphate was obtained from Fisher, and NAD^+ was acquired from Bioworld. All were used without further purification. Rates were normalized using rate for 250 μM cinnamyl alcohol as the standard.

B. MTS-Formazan Assay

Due to the low solubility of long chain NAEs full kinetic assays to determine their kinetic constants could not be performed. As the compounds could only be assayed at relatively low concentrations (<100 μM), the rates of NADH production were very low and could not be confidently determined using the spectrophotometric NADH assay. Instead, a more sensitive method for NADH detection was employed, coupling the NADH production to the reduction of a tetrazolium dye MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) to the

colored formazan with λ_{max} at 490 nm and $\epsilon = 20,800 \text{ M}^{-1}\text{cm}^{-1}$ at pH 9.5 (Fig. 7). PMS (phenazinemethosulfate) was used as an intermediate electron carrier.

Figure 7. MTS-formazan assay for NADH detection.

NAEs with 10 or more carbon atoms in the chain were all assayed for ADH3 activity at the same concentration employing the MTS-formazan assay. The assay conditions were as follows: 100 mM sodium pyrophosphate pH 9.5, 55 μM NAE, 2.5 mM NAD^+ , 150 μM MTS, 8.25 μM PMS, 3 % DMSO and 30 $\mu\text{g/ml}$ ADH3. Decanoyl and lauroylethanolamine were soluble enough to allow the determination of K_M and V_{Max} values as well. All reactions were done at 37 °C and the initial rates were determined by observing the increase in absorbance at 490 nm using JASCO V-530 UV-Vis spectrophotometer. MTS was purchased from Promega and PMS was obtained from TCI America. Rates for cinnamyl alcohol obtained in this manner matched the rates obtained by following NADH production at 340 nm. Rates were normalized using rate for 250 μM cinnamyl alcohol as the standard.

Modeling Experiments

The crystal structure of human ADH3 (PDB ID 1MP0 (41)) was used for grid-based ligand docking. All co-crystallized ligands deemed superfluous for enzyme function were removed from the crystal structure and polar hydrogens were added using AutoDockTools. Charges were then corrected for the requisite zinc ions and bond orders corrected for the co-substrate, NAD^+ . The receptor grid was prepared with a grid point

spacing of 0.2 Å using AutoGrid. The substrates of interest were then prepared using AutoDockTools to define torsions, rotamers, and polar hydrogens. The ligands were then docked into the active site of ADH using AutoDock 4.0 (42-43). All default settings were utilized with the exception of increasing the number of energy evaluations from 2.5×10^4 to 2.5×10^7 .

Product Characterization Experiments

A. HPLC Separation Experiments

Experiments were performed on a HP 1100 Agilent, equipped with a 4-channel solvent mixing system, a quaternary pump, and a deuterium lamp UV detector. All separations were accomplished using a Thermo-Scientific C18 column (4.6 x 250 mm), with temperature regulated at 40 °C. *N*-Benzoylethanolamine and its derivatives were separated using either a gradient of 50 mM sodium phosphate pH 6.0: CH₃CN - 90: 10 to 95:5 over 20 minutes, or isocratic mobile phase of 50 mM sodium phosphate pH 6.0: CH₃CN (90:10 (v/v)) (Fig. B-1). Compounds were detected by following UV absorbance at 230 nm.

Cinnamyl alcohol and its derivatives were separated using gradient of 50 mM sodium phosphate pH 6.0: CH₃CN – 75:25 to 40:60 over 15 minutes, and compounds were detected by monitoring absorbance at 265 nm (Fig B-2).

N-Octanoylglycinal semicarbazone and semicarbazide were separated using gradient of H₂O: CH₃CN - 70:30 to 40:60 over 20 minutes (Fig B-3). The compounds were detected by monitoring absorbance at 210 nm.

B. GC-MS Experiments

GC-MS experiments were performed on Shimadzu GC-MS instrument equipped with a DB-5 (0.25 µm x 0.25 mm x 30 m) column. Compounds were extracted from reaction mixture using either ethyl ether (cinnamyl alcohol and cinnamyl aldehyde) or CH₂Cl₂ (*N*-benzoylethanolamine and its derivatives). Reaction mixture was acidified prior to *N*-benzoylglycine extraction. Solvent was removed and samples were derivatized prior to analysis in order to increase their volatility and signal strength. Aldehyde

derivatization to PFB-oximes was done by dissolving the extracted and dried residue into 90 μ l of CH₃CN, adding 10 μ l of 100 mM PFBHA (*O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine), and heating the solution at 60 °C for 60 minutes (Scheme 4).

Scheme 4. PFBHA derivatization reaction.

N-Benzoylglycine derivatization was done by dissolving the extracted and dried material in 100 μ l of BSTFA (*N,O*-bis(trimethylsilyl)trifluoroacetamide), purging the solution with N₂, and heating it at 90 °C for 15-20 minutes (Scheme 5). Carboxylic group and amide moiety of NAGs can be both be derivatized by this method, as can hydroxyl and amide groups of NAEs.

Scheme 5. BSTFA derivatization reaction.

Derivatized samples (5-10 μ l) were injected into the GC-MS in a splitless manner, with injection temperature at 250 $^{\circ}$ C. Temperature program was modified from Merkler et al. (44). Oven temperature was raised from 55.0 $^{\circ}$ C to 150.0 $^{\circ}$ C, at a rate of 40.0 $^{\circ}$ C/min, held at 150.0 $^{\circ}$ C for 3.6 min, then raised to 300 $^{\circ}$ C at a rate of 10.0 $^{\circ}$ C/min, and finally held at 300 $^{\circ}$ C for 1.0 min. Interface temperature between GC and MS was 280 $^{\circ}$ C, and solvent cut time was 7-9 min. For *N*-benzoylglycinal detection, injection volume was increased to 40 μ l in order to more confidently detect the TMS-benzoylglycine derivative. Solvent cut time was raised to 11.5 min, so as to avoid column overload by the far more concentrated di- and mono-TMS-*N*-benzoylethanolamine derivatives eluting at 10.7 and 11.2 min, respectively. Peak identity was established by comparison of retention times and mass spectra with those of derivatized standards and library spectra.

C. Trapping *N*-acylglycinals with semicarbazide

In order to characterize the reaction products, *N*-acylglycinals, which are unstable at reaction pH, semicarbazide was employed as an aldehyde-trapping reagent. Semicarbazide was added in large excess (9-10 x substrate concentration) to the reaction mix containing 100 mM sodium pyrophosphate pH 9.5, 3-5 mM *N*-benzoylethanolamine, 2.5 mM NAD⁺, and 0.25-1.0 mg/ml ADH3. Formation of *N*-benzoylglycinal semicarbazone was followed by HPLC.

D. *N*-Acylglycinal Semicarbazone Characterization by ¹³C NMR

N-Octanoylglycinal semicarbazone was characterized by following the enzymatic oxidation of [1',2'-¹³C]-*N*-octanoylethanolamine in the presence of semicarbazide by ¹³C NMR. The presence of *N*-octanoylglycinal semicarbazone was established by observing the reduction in intensity of the ¹³C labeled signals in the substrate and the appearance of two ¹³C carbon signals consistent with those of *N*-octanoylglycinal semicarbazone. Peak identity was confirmed by comparison with ¹³C spectrum of synthesized *N*-octanoylglycinal semicarbazone (Fig. A-12). Reaction conditions were as follows: 50 mM sodium pyrophosphate pH 8.0, 2.5 mM [1',2'-¹³C]-*N*-octanoylethanolamine, 2.5 mM NAD⁺, 20 mM semicarbazide HCl, 10% D₂O, and 1.7 mg/ml ADH3. Reaction mix was incubated at 37 $^{\circ}$ C, and ¹³C NMR spectra were taken after 24 hour and 48 hour reaction

time points. Control samples containing all the reagents except for the enzyme were handled and analyzed in the same manner. Experiments were performed on a Varian iNova 400 MHz instrument.

Chapter 3 – Results and Discussion

ADH3 Purification

ADH3 was purified from bovine liver to ~ 90 % homogeneity (Fig. 8), and stored at -80 °C for later use in enzymatic assays. Specific activity of purified ADH3 was 0.53 U/mg, which is somewhat lower than the previously reported values of ~ 3.0 U/mg (38). Relatively low activity is most likely due to the fact that enzyme was purified from liver that had been stored in the freezer for several months, rather than a fresh sample. Molecular weight of ADH3 subunits, calculated based on the R_f values determined from the SDS-PAGE was 38 kDa, which is in good agreement with previously determined values of 40-41 kDa (32).

Figure 8. SDS-PAGE of purified bovine liver ADH3. The outer two lanes contain the Kaleidoscope molecular weight ladder (MWL), and the inner lanes contain purified ADH3.

Kinetics of ADH3-catalyzed NAE Oxidation

A variety of aliphatic NAEs with chain lengths varying from 2 to 20 carbons, as well as several aromatic NAEs were evaluated as substrates for ADH3, in the manner described previously (Fig. 9). All of the tested NAEs were found to be substrates for ADH3, with K_M values decreasing with increase in chain length of the acyl substituent (Table 1). This indicated that increase in substrate size led to tighter binding to the enzyme, or rather that small size impaired the ability to bind to the site tightly, which was in agreement with known substrate preferences of ADH3 (26) and previous crystallographic studies of the enzyme active site (30-32). V_{MAX} followed the same trend, decreasing with increase in substrate size and hydrophobicity. However, decrease in K_M was more dramatic, resulting in overall increase in V_{MAX}/K_M values with increase in substrate chain length (see Table 1). These findings are in agreement with the previous studies, which established the preference of ADH3 for bulky, longer chain alcohol substrates, in contrast with other ADH enzymes (26). While ADH3 could catalyze the oxidation of all tested NAEs, their turnover values were low compared to cinnamyl alcohol, one of the preferred ADH3 substrates (see Tables 1 and 3).

Figure 9. A representative Michaelis-Menten plot for one of the assayed NAEs.

Table 1. Kinetic constants for short- and medium-chain NAEs.

Substrate		K_M (mM)	V_{Max} ($\mu\text{mol}/\text{min}/\text{mg}$)	V_{Max} / K_M
<i>N</i> -acetyethanolamine	R = CH ₃	$(4.5 \pm 0.35) \times 10^2$	1.9 ± 0.059	0.0041
<i>N</i> -propionylethanolamine	R = CH ₃ CH ₂	79 ± 3.8	1.9 ± 0.026	0.024
<i>N</i> -butyrylethanolamine	R = CH ₃ (CH ₂) ₂	46 ± 5.4	1.7 ± 0.052	0.038
<i>N</i> -hexanoylethanolamine	R = CH ₃ (CH ₂) ₄	9.5 ± 0.36	1.6 ± 0.018	0.17
<i>N</i> -octanoylethanolamine	R = CH ₃ (CH ₂) ₆	5.8 ± 0.38	1.5 ± 0.028	0.26
<i>N</i> -decanoylethanolamine	R = CH ₃ (CH ₂) ₈	0.32 ± 0.029	0.57 ± 0.024	1.8
<i>N</i> -lauroylethanolamine	R = CH ₃ (CH ₂) ₁₀	0.033 ± 0.0061	0.14 ± 0.0088	4.3

Kinetic constants could not be determined for NAEs longer than lauroyl due to very low solubility and low reaction rates. Table 2 shows a comparison of rates for two medium-chain (decanoyl and lauroyl) and several long chain NAEs at a single substrate concentration. Rates continue to follow a decreasing trend observed with short and medium chain NAEs, with the exception of myristoylethanolamine which has a significantly lower rate compared to the longer NAEs tested. It is possible that this result is related to the unsaturated bonds present in other higher length NAEs, which may enable tighter binding to the enzyme.

Table 2. Relative ADH3 rates for medium and long-chain NAEs

Substrate		Relative Rates
<i>N</i> -decanoylethanolamine	R = CH ₃ (CH ₂) ₈	1.0
<i>N</i> -lauroylethanolamine	R = CH ₃ (CH ₂) ₁₀	0.57
<i>N</i> -myristoylethanolamine	R = CH ₃ (CH ₂) ₁₂	0.061
<i>N</i> -oleoylethanolamine	R = CH ₃ (CH ₂) ₇ HC=CH(CH) ₇	0.28
<i>N</i> -linoleoylethanolamine	R = CH ₃ (CH ₂) ₇ HC=CHCH ₂ HC=CH(CH) ₄	0.26
<i>N</i> -arachidonoylethanolamine	R = CH ₃ (CH ₂) ₃ HC=CHCH ₂ HC=CHCH ₂ HC=CHCH ₂ HC=CH(CH) ₄	0.24

Among the NAEs with an aromatic substituent, compounds with benzyl group had lower K_M values, higher V_{Max} values, and therefore higher turnover values compared to compounds with a phenyl substituent (see Table 3), indicating that the presence of CH₂

group following the aromatic ring might have contributed to better binding and more efficient catalysis.

Table 3. Kinetic constants for aromatic NAEs and cinnamyl alcohol.

Substrate		K_M (mM)	V_{Max} ($\mu\text{mol}/\text{min}/\text{mg}$)	V_{Max} / K_M
<i>N</i> -benzoylethanolamine	$R = C_6H_5$	5.2 ± 0.52	0.36 ± 0.0098	0.069
<i>N</i> -phenylacetyethanolamine	$R = C_6H_5CH_2$	3.9 ± 0.24	1.2 ± 0.026	0.32
<i>N</i> -benzyloxycarbonylethanolamine	$R = C_6H_5CH_2O$	2.3 ± 0.14	0.81 ± 0.014	0.36
<i>N</i> -(2-Phenoxyacetyl)ethanolamine	$R = C_6H_5OCH_2$	6.3 ± 0.36	0.25 ± 0.0042	0.040
Cinnamyl alcohol	$C_6H_5HC=CHCH_2OH$	0.035 ± 0.0033	4.0 ± 0.058	4.1×10^2

Docking Experiments

NAEs with chain length between 2 and 11 carbons were docked into the human ADH3 crystal structure using AutoDock 4.0 in order to evaluate their relative binding energies (Table 4). Sequence alignment of human and bovine ADH3 using BLAST (45-46) shows that they share 94% identity, with 98 % of residues conserved, making the docking results obtained with the human enzyme applicable to bovine ADH3 as well. Relative binding energies of docked NAEs were found to follow the same general trend as K_M values, decreasing with increase in chain length (Table 4), indicating that tighter binding to the enzyme is at least partly responsible for the observed K_M trend. These findings provide further agreement with previously determined substrate preference (26) and crystallographic data (30-32). Substrates were docked in the enzyme active site with hydroxyl group coordinating with the catalytic Zn ion (Fig. 7), in the same manner as other ADH3 substrates have been shown to bind (31-32).

Table 4. Relative binding energies and K_M values for selected NAEs and cinnamyl alcohol. Binding energies values were calculated using Autodock 4.0, and have a standard error of about ± 2.5 kcal/mol (43).

Substrate	K_M (mM)	Estimated free energy of binding (kcal/mol)
<i>N</i> -acetylethanolamine	$(4.5 \pm 0.35) \times 10^2$	-3.72
<i>N</i> -propionylethanolamine	79 ± 3.8	-4.14
<i>N</i> -butyrylethanolamine	46 ± 5.4	-4.22
<i>N</i> -hexanoylethanolamine	9.5 ± 0.36	-4.34
<i>N</i> -octanoylethanolamine	5.8 ± 0.38	-4.31
<i>N</i> -decanoylethanolamine	0.32 ± 0.029	-4.34
<i>N</i> -lauroylethanolamine	0.033 ± 0.0061	-4.06
<i>N</i> -benzoylethanolamine	5.2 ± 0.52	-4.58
Cinnamyl alcohol	0.035 ± 0.0033	-4.68

Figure 10. *N*-Hexanoylethanolamine docked into the active site of human ADH3 (PDB ID 1MP0). Protein backbone is shown in green, Zn ions are gray spheres, and NAD^+ and *N*-hexanoylethanolamine are shown as stick models with CPK-colored atoms.

Product Characterization

Characterization of the product of the enzymatic reaction was initially attempted by following the reaction progress by HPLC. HPLC analysis of the ADH3-catalyzed *N*-benzoylethanolamine oxidation revealed the presence of a peak consistent with *N*-benzoylglycine as well as a peak corresponding to *N*-benzoylglycinal (Fig. 11). *N*-Benzoylglycine and *N*-benzoylglycinal are the two possible products of the enzymatic oxidation of *N*-benzoylethanolamine. However, ADH3 has not been found to catalyze the dismutation of aldehydes (18). Experiments using *N*-benzoylglycinal as a substrate for ADH3 indicated that ADH3 is not able to catalyze the oxidation of *N*-benzoylglycinal, but that *N*-benzoylglycinal can undergo non-enzymatic dismutation to *N*-benzoylglycine and *N*-benzoylethanolamine under the employed reaction conditions (2.5 mM NAD⁺, pH 9.5-10). HPLC analysis of ADH3-catalyzed cinnamyl alcohol oxidation revealed only peak consistent with cinnamyl aldehyde (Fig. 12), further suggesting that the reaction products of NAE oxidation are *N*-acylglycinals, and that any NAGs observed are produced through non-enzymatic means.

Figure 11. HPLC separation of ADH3-catalyzed *N*-benzoylethanolamine oxidation. Reaction conditions were as follows: 100 mM sodium pyrophosphate pH 9.5, 2.5 mM NAD⁺, 6 mM *N*-benzoylethanolamine, and 2.4 mg/ml ADH3. An aliquot was taken for HPLC analysis after 36 hours of reaction time. Peak at 3.918 was determined to be NAD⁺ derivative formed during prolonged incubation at high pH and was also observed in the control sample containing only NAD⁺ and buffer.

Figure 12. HPLC separation of ADH3-catalyzed cinnamyl alcohol oxidation. Reaction conditions were as follows: 100 mM sodium pyrophosphate pH 9.5, 2.5 mM NAD^+ , 1.75 mM cinnamyl alcohol, and 5 $\mu\text{g/ml}$ ADH3. HPLC analysis was performed after 45 minutes of reaction time.

Further characterization of the product peaks observed by HPLC was conducted using GC-MS. In order to test the validity of the method, the procedure was first used to characterize cinnamyl alcohol reaction product. Derivatization of cinnamyl alcohol reaction extract with PFBHA gave two peaks detected by GC-MS, whose retention times and fragmentation patterns were consistent with those for two isomers of cinnamyl aldehyde PFB-oxime (Fig. 13), thus confirming that the reaction product is cinnamyl aldehyde. However, applying the same method to characterize *N*-benzoylglycinal observed by HPLC proved unsuccessful. Although derivatized standard for *N*-benzoylglycinal-PFB-oxime was successfully characterized using GC-MS (Fig C-3), the compound could not be detected by this method in the reaction extracts. This can be attributed to the very low turnover rates of *N*-benzoyl ethanolamine oxidation compared to cinnamyl alcohol oxidation (Table 3), as well as to the tendency of *N*-benzoylglycinal to undergo oxidation to *N*-benzoylglycine under the reaction conditions. Derivatizing reaction extract with BSTFA resulted in detection of *N,O*-di-TMS-*N*-benzoylglycine (Fig. 14), thus confirming that the peak observed by HPLC is *N*-benzoylglycine.

Line#:1 R.Time:13.713(Scan#:1576)
 MassPeaks:129
 RawMode:Single 13.713(1576) BasePeak:181.05(692137)
 BG Mode:None

Line#:1 R.Time:13.965(Scan#:1617)
 MassPeaks:139
 RawMode:Single 13.965(1617) BasePeak:181.05(1788757)
 BG Mode:None

Figure 13. GC-MS spectra of cinnamyl aldehyde *O*-PFB-oximes. Cinnamyl aldehyde PFB-oximes were detected through GC-MS analysis of cinnamyl alcohol reaction extract that was treated with PFBHA. The two peaks correspond to the two cinnamyl aldehyde *O*-PFB-oxime isomers (*E* and *Z*), which differ in retention times, but have identical fragmentation patterns. See Fig. C-1 for cinnamyl aldehyde *O*-PFB-oxime standard spectra.

Figure 14. GC-MS spectrum of *N,O*-di-TMS-*N*-benzoylglycine. *N,O*-di-TMS-*N*-benzoylglycine peak was detected in derivatized extract of ADH3-catalyzed *N*-benzoylethanolamine reaction. Extraction and GC-MS analysis were performed after 24 hours of reaction time.

In order to detect and characterize the unstable *N*-acylglycinal product, a well-known aldehyde-trapping reagent, semicarbazide, was added to the reaction mixture. Semicarbazide readily reacts with aldehydes and ketones to form semicarbazones (Fig. 15). HPLC analysis of *N*-benzoylethanolamine reaction containing semicarbazide revealed a peak consistent with the peak for *N*-benzoylglycinal semicarbazone standard (Figs.12 and B-1). Further characterization of the semicarbazone derivative was attempted by LC-MS and GC-MS, but proved unsuccessful.

Figure 15. Semicarbazone formation reaction.

Figure 16. HPLC analysis of ADH3-catalyzed *N*-benzoyl ethanolamine oxidation in the presence of semicarbazide. Reaction conditions were as follows: 100 mM sodium pyrophosphate pH 9.5, 2.5 mM NAD⁺, 4 mM *N*-benzoyl ethanolamine and 300 µg/ml ADH3. HPLC analysis was performed after 24 hours of reaction time.

In order to characterize the putative *N*-acylglycinal semicarbazone species detected by HPLC, reaction was analyzed by ¹³C NMR. For the purpose of increased sensitivity, [1',2'-¹³C]-*N*-octanoyl ethanolamine was synthesized and employed as the reaction substrate. Analysis of [1',2'-¹³C]-*N*-octanoyl ethanolamine reaction showed a total of 4 different ¹³C signals – two that matched those for the substrate, at 41 and 60 ppm (Fig. A-9), and two new ¹³C signals, at 40 and 143 ppm. Chemical shifts of the two new signals match those of the corresponding carbon atoms in the *N*-octanoylglycinal semicarbazone standard (Figs. 17 and A-12), thus providing confirmation that the product of the ADH3 catalyzed oxidation is *N*-acylglycinal, in agreement with previously published data (18). Performing the reaction in absence of semicarbazide resulted in

appearance of ^{13}C signals consistent with those of *N*-octanoylglycine (Figs. 18 and A-13). This result was consistent with previous HPLC experiments, which showed that *N*-benzoylglycinal is readily oxidized to *N*-benzoylglycine at basic pH. Control sample with no ADH3 present showed only the two substrate peaks (Fig A-14), confirming there was no detectable oxidation of *N*-octanylethanolamine through non-enzymatic means.

Figure 17. ^{13}C NMR spectrum of ADH3-catalyzed $[1',2'\text{-}^{13}\text{C}]\text{-}N\text{-octanylethanolamine}$ oxidation in the presence of semicarbazide. Reaction conditions were as indicated in Chapter 2, and spectrum was taken after 48 hours of reaction time.

Figure 18. ^{13}C NMR spectrum of ADH3-catalyzed $[1',2'\text{-}^{13}\text{C}]\text{-N-octanylethanolamine}$ oxidation **without semicarbazide**. Reaction conditions were as indicated in Chapter 2 with the exception of semicarbazide, which was omitted. Spectrum was taken after 48 hours of reaction time.

Conclusion

Evaluation of NAEs as ADH3 substrates showed that they can be oxidized by this enzyme, which is known to act on a variety of mid- and long-chain alcohol substrates. In accordance with previous literature reports, the reaction products were shown to be *N*-acylglycinals, which ADH3 was unable to oxidize further to NAGs. Relatively low conversion rates observed for ADH3-catalyzed NAE oxidation make it unlikely that ADH3 plays a significant role in conversion of NAEs to *N*-acylglycinals *in vivo*. Nevertheless, our findings validate the chemistry proposed in the pathway, and further suggest that another ADH enzyme could be responsible for the oxidative degradation of NAEs. Further studies on ADH5 and ADH6 should be completed in order to evaluate the possibility of their involvement in NAE metabolism, as these two enzymes have as of yet been poorly investigated and could potentially play the role of FADH in the conversion

of NAEs to NAGs. Additionally, NAEs could be evaluated as substrates or inhibitors of other ADH enzymes, in order to explore their potential role in alcohol or retinol metabolism.

References Cited

1. Farrell, E. K., and Merkler, D. J. (2008) Biosynthesis, degradation and pharmacological importance of fatty acid amides. *Drug Discov. Today*. 13, 558-568.
2. Schmid, H.H.O., and Berdyshev E. V., (2002) Cannabinoid receptor-inactive *N*-acylethanolamines and other fatty acid amides: metabolism and function. *Prostaglandins, Leukot. Essent. Fatty Acids*. 66, 363-376.
3. Lambert, D. M., and Muccioli, G. G. (2007) Endocannabinoids and related *N*-acylethanolamines in the control of appetite and energy metabolism: emergence of new molecular players. *Curr. Opin. Nutr. Metab. Care*. 10, 735-744.
4. Hansen, H. S., Moesgaard, B., Petersen G., and Hansen, H. H. (2002) Putative neuroprotective actions of *N*-acylethanolamines. *Pharmacol. Ther.* 95, 119-126.
5. Lambert, D. M., Vandervoorde, S., Jonsson, K., and Fowler, C. J. (2002) The palmitoylethanolamide family: a new class of anti-inflammatory agents? *Curr. Med. Chem.* 9, 663-674.
6. Dalle Carbonare, M., Del Giudice, E., Stecca, A., Colavito, D., Fabris, M., D'Arrigo, A., Bernardini, D., Dam, M., and Leon, A. (2008) A saturated *N*-acylethanolamine other than *N*-palmitoyl ethanolamine with anti-inflammatory properties: a neglected story. *J. Neuroendocrinol.* 20 (suppl.1), 26-34.
7. Jonsson, K., Vandervoorde, S., Lambert, D. M., Tiger, G., and Fowler, C. J. (2001). Effects of homologues and analogues of palmitoylethanolamide upon the activation of the endocannabinoid anandamide. *Br. J. Pharmacol.* 133, 1263-1275.
8. Lambert, D. M., DiPaolo, F. G., Sonveaux, P., Kanyonyo, M., Govaerts, S. J., Hermans, E., Bueb, J., Delzenne, N. M., and Tschirhart, E. J. (1999) Analogues and homologues of *N*-palmitoylethanolamide, a putative endogenous CB2 cannabinoid, as potential ligands for the cannabinoid receptors. *Biochim. Biophys. Acta*. 1440, 266-274.

9. Tsuboi, K., Takezaki, N., and Ueda, N. (2007) The *N*-acylethanolamine-hydrolyzing acid amidase (NAAA). *Chem. Biodivers.* 4, 1914-1925.
10. Ueda, N., Tsuboi, K., and Lambert, D. M. (2005) A second *N*-acylethanolamine hydrolase in mammalian tissues. *Neuropharmacology.* 48, 1079-1085.
11. Arafat, E. S., Trimble, J. W., Andersen, R. N., Dass, C., and Desiderio, D. M. (1989) Identification of fatty acid amides in human plasma. *Life Sci.* 45, 1679-1687.
12. Burstein, S. H., Rossetti, R. G., Yagen, B., and Zurier R. B. (2000) Oxidative metabolism of anandamide. *Prostaglandins & other Lipid Mediat.* 61, 29-41.
13. Sugiura, K., Kondo, S., Kodaka, T., Tonegawa, T., Nakane, S., Yamashita, A., Ishima, Y., and Waku, K. (1996). Enzymatic synthesis of oleamide (cis-9, 10-octadecenoamide), and endogenous sleep-inducing lipid, by rat brain microsomes. *Biochem. Mol. Biol. Int.* 40, 931-938.
14. Mueller, G. P., and Driscoll, W. J. (2007) In vitro synthesis of oleoylglycine by cytochrome c points to a novel pathway for the production of lipid signaling molecules. *J. Biol. Chem.* 282, 22364-22369.
15. Driscoll, W. J., Chaturvedi, S., and Mueller, G. P. (2007) Oleamide synthesizing activity from rat kidney – identification as cytochrome c. *J. Biol. Chem.* 282, 22353-22363.
16. Jörnvall, H. and Höög, J. (1995) Nomenclature of alcohol dehydrogenase. *Alcohol Alcohol.* 30, 153-161
17. Yin, S., Han, C., Lee, A., and Wu, C. (1999) Human alcohol dehydrogenase family. *Adv. Exp. Med. Biol.* 463, 265-274.
18. Svensson, S., Lundsjö, A., Cronholm, T., and Höög, J. (1996) Aldehyde dismutase activity of human liver alcohol dehydrogenase. *FEBS Lett.* 394, 217-220.
19. Yin, S., Chou, C., Lai, C., Lee, S., and Han, C. (2003) Human class IV alcohol dehydrogenase: kinetic mechanism, functional roles and medical relevance. *Chem. Biol. Interact.* 143-144, 219-227.
20. Höög, J., Hedberg, J. J., Strömberg, P., and Svensson, S. (2001) Mammalian alcohol dehydrogenases – functional and structural implications. *J Biomed Sci.* 8, 71-76.
21. Duester, G. (2000) Families of retinoid dehydrogenases vitamin A function – production of visual pigment and retinoic acid. *Eur. J. Biochem.* 267, 4315-4324.

22. Jörnvall, H., Höög, J., Persson B., and Parés, X. (2000) Pharmacogenetics of the alcohol dehydrogenase system. *Pharmacology*. 61, 184-191.
23. Yasunami, M., Chen, C., and Yoshida, A. (1999) A human alcohol dehydrogenase gene (ADH6) encoding an additional class of isozyme. *Proc. Natl. Acad. Sci.* 88, 7916-7614.
24. Zheng, Y., Bey, M., Liu, H., and Felder, M. R. (1993) Molecular basis of the alcohol dehydrogenase-negative deer mouse. *J. Biol. Chem.* 268, 24933-24939.
25. Parés, X., and Vallee, B. L. (1981) New human liver alcohol dehydrogenase forms with unique characteristics. *Biochem. Biophys. Res. Commun.* 98, 122-130.
26. Wagner, F. W., Parés, X., Holmquist, B., and Vallee, B. L. (1984) Physical and Enzymatic Properties of a Class III Isozyme of Human Liver Alcohol Dehydrogenase: χ -ADH. *Biochemistry*, 23, 2193-2199.
27. Koivusalo, M., Baumann, M., and Uotila, L. (1989) Evidence for the identity of glutathione-dependent formaldehyde dehydrogenase and class III alcohol dehydrogenase. *FEBS Lett.* 257, 105-9.
28. Holmquist, B., and Vallee, B. L. (1991) Human liver class III alcohol and glutathione dependent formaldehyde dehydrogenase are the same enzyme. *Biochem. Biophys. Res. Commun.* 178, 1371-1377.
29. Lee, S., Wang, M., Lee, A., and Yin, S. (2003) The metabolic role of human ADH3 functioning as ethanol dehydrogenase. *FEBS Lett.* 544, 143-147.
30. Yang, Z., Bosron, W. F., and Hurley, T. D., (1997) Structure of human $\chi\chi$ alcohol dehydrogenase: a glutathione-dependent formaldehyde dehydrogenase. *J. Mol. Biol.* 265, 330-343.
31. Sanghani, P. C., Bosron, W. F., and Hurley, T. D. (2002) Human glutathione-dependent formaldehyde dehydrogenase. Structural changes associated with ternary complex formation. *Biochemistry*. 41, 15189-15194.
32. Sanghani, P. C., Robinson, H., Bosron, W. F., and Hurley, T. D. (2002) Human glutathione-dependent formaldehyde dehydrogenase. Structures of apo, binary, and inhibitory ternary complexes. *Biochemistry*. 41, 10778-10786.

33. Ramaswamy, S., Eklund, H., and Plapp, B. V. (1994). Structures of horse liver alcohol dehydrogenase complexed with NAD⁺ and substituted benzyl alcohols. *Biochemistry*, 33, 5230-5237.
34. Eklund, H., and Brändén, C. (1979). Structural differences between apo- and holoenzyme of horse liver alcohol dehydrogenase. *J. Biol. Chem.* 254, 3458-3461.
35. Colonna-Cesari, F., Perahia, D., Karplus, M., Eklund, H., Brändén, C. I., and Tapia, O. (1986) Interdomain motion in liver alcohol dehydrogenase: structural and energetic analysis of the hinge bending mode. *J. Biol. Chem.* 261, 15273-15280.
36. Sanghani, P. C., Stone C. L., Ray, B. D., Pindel, E. P., Hurley, T. D., and Bosron, W. F. (2000) Kinetic mechanism of human glutathione-dependent formaldehyde dehydrogenase. *Biochemistry*. 39, 10720-10729.
37. Giri, P. R., Linnoila, M., O'Neill, J. B., and Goldman, D. (1989) Distribution and possible metabolic role of class III alcohol dehydrogenase in the human brain. *Brain Res.* 481, 131-141.
38. Pourmotabbed, T., and Creighton, D. J. (1986) Substrate specificity of bovine liver formaldehyde dehydrogenase. *J. Biol. Chem.* 261, 14240-14244.
39. Brown, E. V. (1949) Penilloaldehydes and penaldic acids. In *Chemistry of penicillin*; Clarke, H. T., Johnson, J. R., and Robinson, R., Eds. Princeton University Press: Princeton, NJ. p 482-483.
40. Iyer, V. I., Sheth, G. N., and Subrahmanyam, V. V. R. (1982) Melting behaviour of some pure *N*-acyl amino acids and peptides. *J. Indian Chem. Soc.* 60, 856-859.
41. Sanghani, P. C., Robinson, H., Bennet-Lovsey, R., Hurley, T. D., and Bosron, W. F. (2003) Structure-function relationships in human class III alcohol dehydrogenase (formaldehyde dehydrogenase). *Chem. Biol. Interact.* 143-144, 195-200.
42. Morris, G. M., Goodsell, D. S., Halliday, R.S., Huey, R., Hart, W. E., Belew, R. K. and Olson, A. J. (1998) Automated docking using a Lamarckian genetic algorithm and empirical binding free energy function. *J. Comput. Chem.* 19, 1639-1662.
43. Huey, R., Morris, G. M., Olson, A. J. and Goodsell, D. S. (2007) A semiempirical free energy force field with charge-based desolvation. *J. Comput. Chem.* 28, 1145-1152.

44. Merkler, D. J., Chew, G. H., Gee, A. J., Merkler, K. A., Sorondo, J. O., and Johnson, M. E. Oleic acid derived metabolites in mouse neuroblastoma N₁₈TG₂ cells. *Biochemistry*. 43, 12667-12674.
45. Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 2, 3389-3402.
46. Altschul, S. F., Wootton, J. C., Gertz, E. M., Agarwala, R., Morgulis, A., Schäffer, A. A., and Yu, Y. (2005) Protein database searches using compositionally adjusted substitution matrices. *FEBS J.* 272, 5101-5109.

APPENDICES

Appendix A – NMR Spectra

Figure A-1. ¹H and ¹³C NMR Spectra of N-Butyrylethanolamine in DMSO-d₆. Peak at 3.409 ppm in the ¹H spectrum is due to presence of a small amount of H₂O in the solvent.

Appendix A (Continued)

Figure A-2. ¹H and ¹³C NMR Spectra of N-Hexanylethanolamine in DMSO-d₆.

Appendix A (Continued)

Figure A-3. ^1H and ^{13}C NMR Spectra of *N*-Octanylethanolamine in DMSO-d_6 .

Appendix A (Continued)

Figure A-4. ¹H and ¹³C NMR Spectra of N-Decanylethanolamine in DMSO-d₆.

Appendix A (Continued)

Figure A-5. ¹H and ¹³C NMR Spectra of N-Lauroylethanolamine in DMSO-d₆.

Appendix A (Continued)

Figure A-6. ¹H and ¹³C NMR Spectra of *N*-Myristoylethanolamine in DMSO-*d*₆. Peak at 3.280 ppm in the ¹H spectrum is due to presence of a small amount of H₂O in the solvent.

Appendix A (Continued)

Figure A-7. ¹H and ¹³C NMR Spectra of N-Oleoylethanolamine in DMSO-d₆.

Appendix A (Continued)

Figure A-8. ^1H and ^{13}C NMR Spectra of *N*-Benzoylethanolamine in DMSO-d_6 . Peak at 3.398 ppm in the ^1H spectrum is due to presence of a small amount of H_2O in the solvent.

Appendix A (Continued)

Figure A-9. ¹H and ¹³C NMR Spectra of [1',2'-¹³C]-N-Octanylethanolamine in DMSO-d₆. Peak at 3.287 ppm in the ¹H spectrum is due to presence of a small amount of H₂O in the solvent. ¹³C-labeled atoms are denoted by *.

Appendix A (Continued)

Figure A-10. ^1H and ^{13}C Spectra of *N*-Benzoylglycinal in CDCl_3 . Peak at 1.598 ppm in the ^1H spectrum is due to presence of a small amount of H_2O in the solvent.

Appendix A (Continued)

Appendix A (Continued)

Figure A-12. ¹H and ¹³C NMR Spectra of N-Octanoylglycinal semicarbazone in DMSO-d₆. Peak at 1.598 ppm in the ¹H spectrum is due to presence of a small amount of H₂O in the solvent. Signal for C atom marked by † is obscured by the DMSO solvent peak in the ¹³C spectrum.

Appendix A (Continued)

Figure A-13. ¹H and ¹³C NMR Spectra of *N*-Octanoylglycine in DMSO-d₆.

Appendix A (Continued)

Figure A-14 . ^{13}C NMR spectrum of $[1',2'\text{-}^{13}\text{C}]$ -*N*-octanylethanolamine control sample without ADH3.

Appendix B – HPLC Spectra

(a)

(b)

Figure B-1. HPLC separation for *N*-benzoylethanolamine and its derivatives. (a) Mobile phase: gradient of 90:10 (50 mM sodium phosphate pH 6.0: CH₃CN) to 95:5 over 20 minutes. (b) Mobile phase: 90:10 (50 mM sodium phosphate pH 6.0: CH₃CN).

Appendix B (Continued)

Figure B-2. HPLC separation of cinnamyl alcohol and its derivatives.

Figure B-3. HPLC separation of semicarbazide and *N*-octanoylglycinal semicarbazone.

Appendix C – GC-MS Spectra

Line#:1 R.Time:13.519(Scan#:896)
 MassPeaks:169
 RawMode:Single 13.519(896) BasePeak:181.00(3492478)
 BG Mode:None

Line#:1 R.Time:13.766(Scan#:936)
 MassPeaks:174
 RawMode:Single 13.766(936) BasePeak:181.00(5405935)
 BG Mode:None

Figure C-1. GC-MS Spectra of cinnamyl aldehyde *O*-PFB-oxime standard.

Appendix C (Continued)

Line#:1 R.Time:11.923(Scan#:475)
 MassPeaks:187
 RawMode:Single 11.923(475) BasePeak:105.20(17514424)
 BG Mode:None

Spectrum

Line#:1 R.Time:12.256(Scan#:529)
 MassPeaks:140
 RawMode:Single 12.256(529) BasePeak:105.15(9270092)
 BG Mode:None

Figure C-2. GC-MS spectra of TMS-derivatized *N*-benzoylglycine standard.

Appendix C (Continued)

Figure C-3. GC-MS Spectrum of *N*-benzoylglycinal *O*-PFB-oxime standard. *E* and *Z* isomers are not resolved.